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Nonradioactive Colony Hybridization Assay for Detection and Enumeration of Enterotoxigenic *Clostridium perfringens* in Raw Beef

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A DNA probe endolabeled with digoxigenin by PCR was developed to detect and enumerate enterotoxigenic *Clostridium perfringens* in raw beef. After 2 h of hybridization, membranes were developed by using an anti-digoxigenin-alkaline phosphatase conjugated antibody. The resulting chromogenic reaction allowed us to detect and enumerate ≤10 CFU of *C. perfringens* per g.

Clostridium perfringens type A is considered one of the most common human food-borne pathogens in the United States. This organism is acquired through the consumption of contaminated meat and poultry products (15). The pathological effects associated with *C. perfringens* food poisoning have been linked to the production of an enterotoxin (5, 7, 16, 20, 25). Food poisoning caused by *C. perfringens* typically requires the ingestion of approximately 10^6 to 10^7 viable vegetative cells per g of food, followed by an incubation period of 8 to 12 h. During this period, the cells undergo sporulation in the small intestine, and large amounts of enterotoxin are released (8).

Routine identification of *C. perfringens* in food samples requires characterization by lengthy procedures, which take more than 48 h and involve the use of selective enrichment medium, biochemical tests, and final confirmation of enterotoxigenic strains by culturing in Duncan-Strong sporulation medium combined with serological analysis (9). The previously described methods for detecting enterotoxigenic *C. perfringens* in food samples include enzyme immunoassays (2, 3, 12, 21); combinations of serotyping, bacteriocin, and plasmid analyses (13); and gene probe methods (23). These techniques have been used in detection and identification schemes following cultivation and isolation of the target organism, which increases the time required to obtain a positive identification. To our knowledge, direct testing of food samples for the presence of enterotoxigenic *C. perfringens* has not been reported previously.

In this paper we describe the use of a simplified method to specifically detect and enumerate enterotoxigenic *C. perfringens* strains in raw beef. The nonisotopic colony hybridization method described below is sensitive enough to detect ≤10 CFU/g in the presence of a heterogeneous bacterial flora containing approximately 10^6 CFU/g.

C. perfringens NCTC 8238, NCTC 8239, and ATCC 10288 were used for enterotoxin detection experiments. The nonenterotoxigenic organisms *C. perfringens* ATCC 3624 and FD-1 were used as negative controls; the latter two strains were kindly provided by R. Labbe (University of Massachusetts, Amherst). The potential for enterotoxin production and the presence of the enterotoxin gene were confirmed by a reversed passive latex agglutination assay (PET-RPLA; Oxoid, Inc., Columbia, Md.) and by PCR amplification, respectively. In the latter procedure, the oligonucleotide primers CPEPS (TGTA GAATATGGATTTGGAAT) and CPENS (AGCTGGGTTT GAGTTTAATGC) (18, 24) were used to amplify the 364-bp enterotoxin fragment (data not shown). Stock cultures were maintained in cooked-meat medium (Difco Laboratories, Detroit, Mich.) stored at 4°C. Vegetative cell cultures were grown by inoculating 0.1-ml portions of the stock culture into 10-ml portions of freshly prepared fluid thioglycollate medium (Difco Laboratories). The inoculated medium was heat shocked at 75°C for 20 min and then incubated aerobically at 37°C for 18 h. To enumerate vegetative cells, the cultures were serially diluted in 0.1% (wt/vol) peptone water and plated onto tryptose-sulfite-cycloserine (TSC) agar; each of the resulting preparations was overlaid with an additional 10 ml of TSC agar (11). The plates were incubated overnight at 37°C in anaerobic jars (BBL GasPack Anaerobic Systems, Beckton Dickinson, Cockeysville, Md.). The following *C. perfringens* strains and other bacterial isolates were used to assess gene probe specificity: *C. perfringens* NCTC 8238, NCTC 8239, ATCC 10388, ATCC 3624, and FD-1; *Clostridium sporogenes* ATCC 3854, FDA 4411, FDA 4434, PA 3674, and WB-5; *Clostridium botulinum* ATCC 7844; *Clostridium tyrobutyricum* ATCC 25755; *Escherichia coli* 90-0105 (serotype O26:H11), Ent C 9490 (serotype O157:H7), 933^+ (serotype O157:H7), and 933^- (serotype O157:H7); *Listeria innocua* SH6-VI; *Listeria monocytogenes* Scott A; *Pseudomonas fluorescens* ATCC 17816; *Salmonella dublin* USDA; *Salmonella enteritidis* USDA; *Shewanella putrefaciens* ATCC 8071; *Shigella flexneri* 5348; *Shigella sonnei* ATCC 20014; and *Yersinia enterocolitica* O121. All of these organisms except *C. perfringens* ATCC 3624 and FD-1 were obtained from the U.S. Department of Agriculture Eastern Regional Research Center Culture Collection; *C. perfringens* ATCC 3624 and FD-1 were obtained from R. Labbe.

Oligonucleotide sequences CPEPS and CPENS (18) were used in the PCR to amplify a 364-bp digoxigenin-labeled probe internal to the *C. perfringens* enterotoxin gene. Each PCR mixture (final volume, 100 μl) contained 200 μM dATP, 20 μM dCTP, 20 μM dGTP, 167 μM dTTP, 33 μM digoxigenin-11-dUTP (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), each primer at a concentration of 0.5 μM, and 500 to 850 ng of strain NCTC 8239 template genomic DNA purified by the method of Saito et al. (18). PCR ampli-

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fication was performed by using a model PTC-100 programmable thermal controller (MJ Research, Inc., Watertown, Mass.). Each of the 30 cycles of PCR amplification consisted of denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min, and DNA extension at 74°C for 2 min, with cycle extension of 3 s per cycle. After 30 cycles, a final extension step was performed by keeping the tubes at 74°C for 10 min and then cooling them to 4°C. The amplified products were detected by electrophoresing 10 to 15 μl of the reaction mixture through a 2% agarose gel with Tris-acetate-EDTA buffer and then staining the gel with ethidium bromide (14). The concentration of the amplified products was also determined by measuring the A_{260}.

Ground beef was obtained from local retail markets. Individual 20-g portions were aseptically weighed, placed into filter stomacher bags (type SFB-0410; Spiral Biotech, Bethesda, Md.), and inoculated with 1-ml portions of the appropriate dilutions of *C. perfringens* cell suspensions to obtain 1, 2, 3, 4, 5, or 6 $\log_{10}$ CFU/g. Meat samples were also inoculated with 1-ml portions of sterile 0.1% (wt/vol) peptone water as a second negative control in addition to enterotoxin-negative strains ATCC 3624 and FD-1, which were added at a concentration of 10^6 CFU/g. Each sample was diluted with 20 ml of filter-sterilized phosphate-buffered saline (pH 7.4) containing 0.1% Tween 80 (Sigma Chemical Co., St. Louis, Mo.). The beef samples were homogenized for 1 min with a Stomacher Lab-Blender model 400 apparatus (Tekmar Co., Cincinnati, Ohio). The meat homogenates (7 to 10 ml) were transferred to sterile 15-ml conical screw-cap tubes (Sarstedt, Inc., Princeton, N.J.) and centrifuged at 500 rpm for 5 min with a Sorvall model GLC-1 centrifuge (Du Pont Co., Wilmington, Del.). Aliquots (1 ml) of the supernatant fluids were filtered under a vacuum through cellulose nitrate filters (model 130 analytical filter units; Nalgene Co., Rochester, N.Y.) along with 5-ml portions of sterile 0.1% (wt/vol) peptone water that were added to uniformly disperse the cells on the filter surfaces. The membranes were then transferred, with the inoculated side facing up, onto the surfaces of TSC agar plates. The plates were placed in anaerobic jars (BBL GasPack Anaerobic Systems) and incubated at 37°C. After incubation for 4, 6, and 18 h, the membranes were removed from the agar surfaces and placed on 3MM chromatography paper (Whatman, Hillsboro, Oreg.) saturated with a denaturing solution (0.5 M NaOH, 1.5 M NaCl). After incubation for 15 min at room temperature the membranes were transferred to a second piece of 3MM paper saturated with a neutralization solution (1.0 M Tris-HCl [pH 8.0], 1.5 M NaCl) and incubated for another 15 min at room temperature. The bacterial DNA was fixed to the cellulose nitrate membranes by the method of Buluwela et al. (1). In addition, duplicate membranes were placed on 3MM paper after the neutralization step and baked at 80°C for 2 h in a conventional oven to fix the bacterial DNA. The membranes were then transferred to 50-ml conical centrifuge tubes (Sarsted, Inc.) and hybridized for 2 h at 65°C with the digoxigenin-labeled probe at a final concentration of 20 ng/ml. The membranes were then transferred to petri plates (15 by 100 mm; Fisher Scientific Co., Malvern, Pa.) for colorimetric development with a digoxigenin-DNA detection kit as recommended in the protocol of the manufacturer (Boehringer Mannheim Biochemicals).

C. perfringens colonies containing the enterotoxin gene were counted visually by using the colony hybridization technique described above. The numbers of vegetative *C. perfringens* cells in the inoculated meat samples were also determined by plating appropriate 10-fold dilutions of the corresponding meat supernatant fluids onto TSC agar. As described above, the diluted samples were plated in duplicate on TSC agar by using a model D spiral plater (Spiral Systems, Cincinnati, Ohio) and also by inoculating 0.1-ml portions of the meat supernatant fluids onto TSC agar.

The numbers of bacteria in the background bacterial flora present in raw ground beef were also determined. Uninoculated meat supernatant fluids were serially diluted and plated onto brain heart infusion medium and plate count agar (Difco Laboratories) by using a spiral plater and also by inoculating 0.1-ml portions of the meat supernatant fluids as described above. The background microflora consistently contained around 10^6 CFU/g.

Confirmation of the presence of enterotoxigenic *C. perfringens* strains by the conventional reverse passive latex agglutination test has proven to be difficult for strains that sporulate poorly in culture media (4, 10, 17). In the procedure described in this paper we combined a membrane filtration method with a shortened nonisotopic colony hybridization technique. This assay was designed to directly test raw meat specimens for the presence of *C. perfringens* strains harboring the enterotoxin gene without the need for bacterial isolation or sample extraction steps. A 364-bp sequence, which has been described previously by Saito et al. (18), was selected as the target for detection with a digoxigenin-labeled DNA probe (6, 22). The specificity of the gene probe which we used was evaluated with a heterogeneous group of bacterial species in pure culture (see above), and no cross-reactions were detected by the colony hybridization procedure when either DNA fixation protocol was used (data not shown).

Incubation periods of 4, 6, and 18 h were assessed to determine the shortest feasible membrane incubation time before hybridization with the digoxigenin-labeled probe. Incubation for 6 h provided a weak but discernable signal, while shorter incubation times did not provide a detectable signal. Overnight incubation provided the most reproducible colony counts, resulting in a total assay time of 2 days. Although prolonged incubation also permitted the normal bacterial flora present in ground beef to grow, the probe exhibited specificity for detection of the *C. perfringens* enterotoxin A gene exclusively.

The two DNA fixation procedures assessed in this study resulted in satisfactory fixation of the bacterial nucleic acids. The microwave fixation method resulted in faster modification without compromising the sensitivity of the assay. The hybridization reactions were performed at 65°C for 2 h at a gene probe concentration of 20 ng/ml. This short incubation period was sufficient to provide a strong hybridization signal consistently. Pre-incubation of the substrate solution at 37°C and signal development at 37°C resulted in development times ranging from 5 min for the strongest signals to a maximum incubation period of 30 min. Development of the hybridization signal with an antidigoxigenin antibody conjugated to an alkaline phosphatase permitted visualization of the bound probe as a blue precipitate when nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate were used as the substrates. No background interference was observed in the presence of the nonspecific bacterial flora normally present in raw ground beef, whose concentration was estimated to be approximately 10^6 CFU/g by the plate count method (Fig. 1). In addition, meat lipids present in the raw beef supernatants did not interfere with the hybridization reaction or the signal development procedure. These lipids are usually responsible for nonspecific binding of the gene probe or detection antibody to the cellulose membrane, resulting in increased background values and poor specificity. The negative controls, which included uninoculated beef samples and samples inoculated with enterotoxin-negative strains FD-1 and ATCC 3624, exhibited

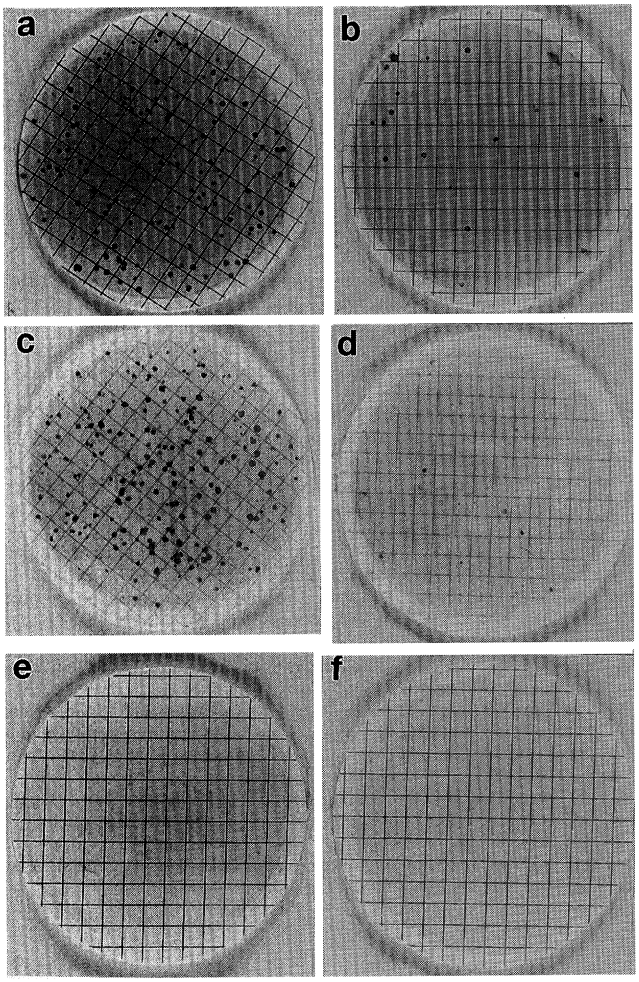

FIG. 1. Representative filters showing colony hybridization of meat supernatants probed with the digoxigenin-labeled enterotoxin gene probe. (a and b) Enterotoxigenic *C. perfringens* NCTC 8238. (c and d) Enterotoxigenic *C. perfringens* NCTC 8239. The filter membranes in panels b and d correspond to 10-fold dilutions of the meat supernatants shown in panels a and c, respectively. (e) Enterotoxin-negative *C. perfringens* FD-1 (10^2 CFU/g, as determined by the anaerobic plate count method). (f) Negative control for background bacterial flora containing no enterotoxigenic *C. perfringens* cells.

TABLE 1. *C. perfringens* vegetative cell counts obtained by plating on TSC agar and by the colony hybridization technique[a]

Strain	No. of cells inoculated into raw beef ($\log_{10}$ CFU/ml)[b]	No. of cells ($\log_{10}$ CFU/g) as determined by:	
		TSC agar method	Gene probe method[c]
NCTC 8238	7.11	5.88	6.34
		6.18	6.30
NCTC 8239	7.70	5.72	6.16
		5.72	6.43
		0[d]	5.00
	7.76	6.79	6.95
	7.90	6.33	6.52
ATCC 10288	8.08	6.37	6.55
		6.37	6.49
FD-1	8.20	6.54	NR[e]
		6.83	NR
ATCC 3624	8.90	6.60	NR
		6.65	NR

[a] The data are the means of the values obtained in two experiments, each performed in duplicate.
[b] Colony counts for 18-h cultures in thioglycollate broth.
[c] Colony hybridization counts. The number of *C. perfringens* cells determined by the gene probe method was significantly higher ($P < 0.05$) than the number of cells determined by the TSC agar plate count method for all strains.
[d] Dilution-plated preparation exceeded the detection limit of the anaerobic plate count method.
[e] NR, no reaction.

no reactions, as reflected by the absence of a hybridization signal in the developed membranes (Fig. 1). The commercial cellulose nitrate filter units used in this study provided efficient fixation of the bacterial nucleic acids when we used treatments such as alkaline denaturation of bacterial cells, microwaving, and a temperature of 80°C. The successful use of these filters in colony hybridization experiments demonstrated that they can be used for the direct enumeration method described in this paper and that cultivation of the test sample and the subsequent colony transfer steps are not needed. Slight variations in the background intensities were observed with different cellulose nitrate filter lots and with different ground beef samples. Enterotoxin-negative strains of *C. perfringens* and uninoculated beef samples provided adequate controls for these background variations.

Using the nonradioactive gene probe detection method, we obtained enough resolution to provide a quantitative assessment of the presence of *C. perfringens* strains harboring the enterotoxin gene. Table 1 shows the bacterial colony counts obtained for *C. perfringens* strains in the spiked meat samples when we used the conventional plate count method and the gene probe procedure developed in this study. Enumeration by the latter procedure was more sensitive than enumeration by the plate count method. When raw beef samples were inoculated with low levels of *C. perfringens* cells containing the enterotoxin gene, the gene probe procedure detected less than 10 CFU/g in the presence of a nonreactive bacterial background concentration of approximately 10^6 CFU/g. Such low cell densities of *C. perfringens* ($\leq$10 CFU/g) usually could not be detected by the plate count procedure.

Data obtained by the anaerobic plate count method and by the colony hybridization method (Table 1) were compared by performing a paired *t* test (19). For statistical purposes, bacterial counts that were not detectable by either method were given a value of zero for the paired *t* test computations. Our statistical analysis showed that the colony hybridization procedure resulted in significantly higher ($P < 0.05$) counts than the plate count method.

In conclusion, the nonisotopic colony hybridization technique was more sensitive than conventional cultivation methods and provided a quantitative assessment of the presence of potentially enterotoxigenic strains of *C. perfringens* as determined by the presence of the enterotoxin A gene. No specific enrichment, selective plating, or sample extraction steps were required for the detection assay; thus, the time-consuming manipulations associated with more conventional methods for cultivation and identification were not necessary. The detection assay was specific for detection of the clostridial enterotoxin A gene in a background flora containing ~10^6 CFU/g of raw beef. While the conventional plate count detection limit is 10 CFU/g, the technique described above was more sensitive, as shown by the higher level of recovery of *C. perfringens* cells. The total detection time was 48 h, with sample processing, membrane filtration, and cultivation on TSC agar occurring on the first day and colony hybridization and enumeration occurring on the second day. These characteristics make the assay

suitable for routine use in diagnostic applications when large numbers of samples must be processed rapidly.

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